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Clathrin Adaptor AP-2 Is Essential for Early Embryonal Development

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The heterotetrameric adaptor protein (AP) complexes AP-1, AP-2, AP-3, and AP-4 play key roles in transport vesicle formation and cargo sorting in post-Golgi trafficking pathways. Studies on cultured mammalian cells have shown that AP-2 mediates rapid endocytosis of a subset of plasma membrane receptors. To determine whether this function is essential in the context of a whole mammalian organism, we carried out targeted disruption of the gene encoding the μ2 subunit of AP-2 in the mouse. We found that μ2 heterozygous mutant mice were viable and had an apparently normal phenotype. In contrast, no μ2 homozygous mutant embryos were identified among blastocysts from intercrossed heterozygotes, indicating that μ2-deficient embryos die before day 3.5 postcoitus (E3.5). These results indicate that AP-2 is indispensable for early embryonic development, which might be due to its requirement for cell viability.

The adaptor protein (AP) complexes AP-1, AP-2, AP-3, and AP-4 are components of protein coats that play key roles in transport vesicle formation and cargo selection in post-Golgi trafficking pathways (21, 29, 38). AP complexes consist of four subunits: two large (α, γ, δ, or ε, and β1–4), one medium (μ1–4), and one small (σ1–4). Each of these subunits fulfills a specific function within the complex. The μ subunits, in particular, recognize YXXΦ-type (Y is tyrosine, X is any amino acid, and Φ is a bulky hydrophobic amino acid), tyrosine-based-sorting signals present in the cytosolic domains of transmembrane proteins, thus mediating the selective capture of these proteins into transport vesicles. AP-2 is by far the best characterized of the AP complexes. Its four subunits are named α, β2, μ2, and σ2; μ2 binds YXXΦ-type signals with the highest affinity and broadest specificity among all the μ subunits. Morphological and biochemical evidence has implicated AP-2 in the rapid endocytosis of cell surface receptors and other plasma membrane proteins (45).

AP-2 is thought to be recruited from the cytosol to the inner leaflet of the plasma membrane by virtue of interactions of its α subunit with membrane-embedded phosphatidylinositol 4,5-bisphosphate (10). μ2 then gets serine phosphorylated by the adaptor-associated kinase, AAK1 (6, 37), and binds phosphatidylinositol 4,5-bisphosphate (15, 40), probably leading to a conformational change that exposes the binding site for YXXΦ-type endocytic signals. This results in the capture of endocytic receptors and their ligands. In parallel, recruitment of clathrin to β2 (42) and of a cohort of accessory proteins to α (35, 44) promotes the formation of clathrin-coated vesicles that bud from the plasma membrane and deliver their cargo to endosomes (4).

Despite the detailed understanding of the molecular mechanisms of AP-2 function, only recently has its physiological role been directly assessed in cultured cells using dominant-negative (5, 31, 35) and RNA interference (RNAi) (13, 16, 18, 24, 28) approaches. These studies have shown that AP-2 is indeed required for the rapid internalization of the transferrin receptor (5, 13, 16, 18, 24, 28, 31) as well as a population of lysosome-associated membrane proteins that traffic via the plasma membrane (18), all of which contain YXXΦ sorting signals that bind to μ2 (7, 33, 45). In contrast, AP-2 appears to be less important for the endocytosis of epidermal growth factor receptor and low-density lipoprotein receptor (5, 28; but also see reference 16), which rely on other types of signals for endocytosis. A notable finding from these studies is that depletion of AP-2 by RNAi of cultured cells did not cause apparent loss of viability over the limited time span of the experiments. However, the RNAi-treated cells still contained small amounts of residual AP-2, which could have been sufficient to sustain cell viability. Meanwhile, in vivo studies of AP-2 function have been demonstrated in the nematode *Caenorhabditis elegans* (12, 20, 43) and the fruit fly *Drosophila melanogaster* (11). Those studies showed that AP-2 plays a critical role in embryonic development.

To address the requirement for AP-2 in the context of a whole mammalian organism, we carried out a targeted disruption of the gene encoding μ2 in mouse. We found that the heterozygous μ2 mutant mice are viable and phenotypically normal, but the homozygous μ2 mutant embryos die very early during development. Indeed, no homozygous mutant embryos were found among the blastocysts from intercrossing of heterozygotes, indicating that μ2-deficient embryos die before day 3.5 postcoitus (E3.5). Our study thus provides the first demonstration that AP-2 is essential for early embryonic

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development of a mammal, most likely due to its requirement for cell viability.

MATERIALS AND METHODS

Animals. All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals, at the NIH, RIKEN, and Kanazawa University.

Antibodies. A monoclonal antibody to mouse CD63 was kindly provided by Toshio Hirano (RIKEN). Polyclonal antibody against μ2 was prepared in our laboratory (2). The following antibodies were purchased from commercial sources: monoclonal antibody to α-adaptin (Transduction Laboratories), monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; CHEMICON), anti-mouse and anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase (Biosource), and Alexa-Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes).

Cells and cell culture. Mouse embryonic fibroblasts (MEFs) from $\mu2^{+/-}$ and wild-type littermates were prepared from E13.5 or E14.5 embryos resulting from the mating of $\mu2^{+/-}$ mice with wild-type mice. MEFs were obtained separately from each fetus. MEFs were placed on a gelatinized plastic dish and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Sigma), 1 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 U/ml streptomycin, and 100 U/ml penicillin. Genotyping of each MEF was done by PCR on tail DNA as described below. Embryonic stem (ES) cells were cultured as described previously (14) in DMEM (Gibco) containing 15% FBS (HyClone), 1 mM L-glutamine, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 U/ml streptomycin, 100 U/ml penicillin, and 1,000 U/ml ESGRO murine leukemia inhibitory factor (Gibco).

Construction of the targeting vector. The μ2 targeting vector consisted of a 1.3-kb PCR fragment corresponding to intron 2, a neomycin phosphotransferase gene (*neo*) expression cassette from PGKneopA (1), a 5.5-kb HindIII-SacI fragment containing exon 12, and a herpes simplex virus thymidine kinase (HSV-tk) gene from pICR/MC1-TK (22). The *neo* cassette was ligated in the opposite transcriptional direction from the μ2 gene and replaced a 3.7-kb fragment containing exons 3 to 11. The vector was linearized at the 5′ end of the 1.3-kb fragment by NotI digestion and introduced by electroporation into R1 ES cells as described previously (22). After selection with G418 (Life Technologies) and ganciclovir (Syntex), surviving colonies were screened for the homologous integration event by Southern blot analysis using a 0.5-kb HindIII-PstI fragment (5′ probe in Fig. 1A) and a 1-kb SacI-HindIII fragment (3′ probe in Fig. 1A), and ES clones with the genotype $\mu2^{+/-}$, resulting from homologous integration of the targeting vector, were obtained.

Targeting of the μ2 gene in ES cells and generation of μ2 mutant mice. Two ES clones were injected into C57BL/6 blastocysts to obtain chimeric mice. The resulting chimeras were bred with C57BL/6 mice, and offspring derived from the ES cells were identified by their agouti coat color and further analyzed for the presence of the $\mu2^{+/-}$ genotype by Southern blot analysis of tail DNA. $\mu2^{+/-}$ mice were intercrossed, and offspring were genotyped by PCR of tail DNA to identify those wild type, heterozygous, and homozygous for the μ2-targeted allele. PCR for identifying the targeted allele was performed using the following primers: sense no. 1 (5′-CGGTATCGCCGCTCCCGATTCGCAGCGCAT-3′) and antisense no. 2 (5′-GTCTACAGAGATGACATCGGGTAAGTCCC-3′). Thermal cycling was carried out for 35 cycles, with denaturation at 98°C for 15 s, annealing at 65°C for 2 s, and extension at 74°C for 30 s in a final reaction volume of 25 μl. PCR for the wild-type allele was performed by a nested-PCR technique using the following primers: for the first-round PCR, sense no. 3 (5′-GCTCATATACGAGCTGCTGGATGGTGAGAC-3′) and antisense no. 4 (5′-GTAGCCAAAGTCCAGAATCTCTGTAAAAAG-3′); and for the second-round PCR, sense no. 5 (5′-TGTAGGTGATTCCTGTACAGCACCAGGACC-3′) and antisense no. 4. The reaction conditions were as follows: for the first round, denaturation at 94°C for 30 s, annealing at 58.5°C for 15 s, and extension at 74°C for 30 s for 40 cycles in the final reaction volume of 50 μl; for the second round, 1 μl of the amplified product from the first-round reaction was used as the template for the second-round reaction with denaturation at 94°C for 30 s, annealing at 58.5°C for 15 s, and extension at 74°C for 30 s for 40 cycles in a final reaction volume of 25 μl.

Genotyping of embryos. $\mu2^{+/-}$ male and female mice were mated and checked for copulation plugs. Embryos were flushed out with phosphate-buffered saline (PBS) from the uteri of plugged females at E3.5. The flushed embryos were heated at 96°C for 10 min before genotyping. Genotyping of embryos was performed using a nested PCR. The following primers were used to detect the targeted allele: for the first round; sense no. 6 (5′-AAACTGAATTGCCTCTTTGCATCTTTTCCC-3′) and antisense no. 7 (5′-CATGGCGATGCCTGCTTGCCGAATATCATG-3′); and for the second round, sense no. 6 and antisense no. 8 (5′-TTGCTGAAGAGCTTGGCGGCGAATGGGCTG-3′). The reaction conditions were as follows: for the first-round, denaturation at 94°C for 30 s, annealing at 58.5°C for 15 s, and extension at 74°C for 30 s for 40 cycles in a final reaction volume of 50 μl; and for the second round, 1 μl of the amplified product from the first-round reaction was used with denaturation at 94°C for 30 s, annealing at 61.5°C for 2 s, and extension at 74°C for 30 s for 40 cycles in a final reaction volume of 25 μl. PCR for detecting the wild-type allele was carried out as described above for tail DNA.

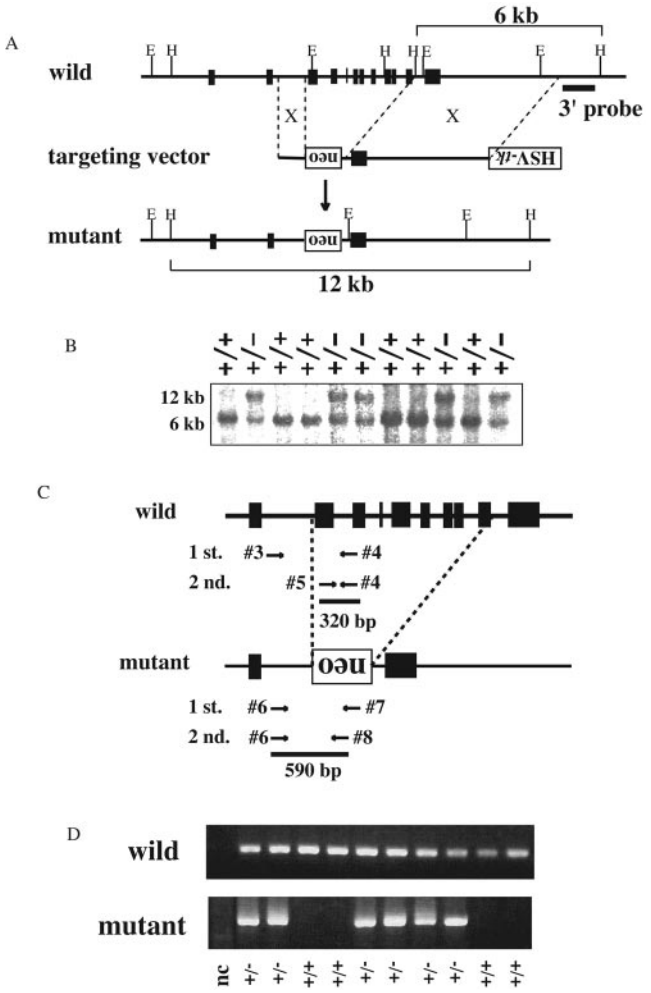

FIG. 1. Establishment of μ2 mutant mice. (A) Schematic representation of the genomic structure of wild-type mice (upper), the targeting vector (middle), and mutant mice (lower). Exons are indicated by filled boxes. Restriction enzyme sites are denoted as follows: E, EcoRI; H, HindIII. The 3′ probe detects a 12-kb band for the mutant allele and a 6-kb band for the wild-type allele in HindIII digestion. (B) Southern blot analysis of littermates from heterozygote intercrossing. The resulting genotypes are indicated over each lane. Note that no homozygous mutants (i.e., yielding only the upper band) were obtained. (C) Schematic representation of the blastocyst genotyping done by nested PCR. The positions of the first- and second-round primers for both genotypes are depicted. (D) Genotyping of littermates by nested PCR. The resulting genotypes are indicated below each lane. Distilled water was used as a template for the negative control (nc; lane 1). Note that no homozygous mutant blastocysts were detected.

Preparation of MEF protein lysate and Western blot analysis. MEFs were washed twice with ice-cold PBS and ice-cold radioimmunoprecipitation assay buffer (0.1% sodium dodecyl sulfate, 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitors [240 μg/ml

TABLE 1. Genotypes of progeny from heterozygous intercrossing

Stage (*n*)	No. (%) with genotype[a]		
	+/+	+/−	−/−
Newborn P28 (133)	40 (30)	93 (70)	0 (0)
Embryo E10.5 (16)	5 (34)	11 (66)	0 (0)
Blastocyst E3.5 (65)	17 (26)	48 (74)	0 (0)

[a] Genotyping of each developmental stage was performed by PCR. A homozygous mutant was not observed. The frequency departs from the expected ratio of 1:2:1 for +/+ versus +/− versus −/− at each stage.

pABSF, 2 μg/ml aprotinin, 157 μg/ml benzamidine hydrate, 10 μg/ml leupeptin, 10 μg/ml chymostatin, and 10 μg/ml pepstatin A]) were added. The cell lysate was recovered by using a cell scraper and passed through a 21-gauge needle four times. Lysis of cells was completed after a 30-min incubation on ice. The lysates were cleared by centrifugation for 15 min at 13,000 rpm in an Eppendorf centrifuge at 4°C. The resulting supernatants were used for Western blot analysis. Protein concentration in the cell lysates was determined using the bicinchoninic acid assay (Pierce). Signals were detected by SuperSignal West Dura or Pico (Pierce) and visualized and quantified by a LAS-3000mini and Image Gauge (Fujifilm, Japan).

Analysis of cell surface receptor internalization. MEFs cultured on plastic dishes were washed twice with PBS and detached with trypsin-EDTA (Gibco). Cells were washed and resuspended in ice-cold fluorescence-activated cell sorter (FACS) buffer (PBS containing 0.1% bovine serum albumin) and incubated with antibody to CD63 (1:50) for 1 h on ice. After washing with ice-cold FACS buffer, cells were incubated at 37°C for 0, 2, or 5 min in DMEM (Gibco) containing 2% FBS (Sigma), 1 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 U/ml streptomycin, 100 U/ml penicillin, and 20 mM HEPES. Cells were washed twice with ice-cold FACS buffer and incubated with Alexa Fluor 488 anti-rat IgG for 30 min on ice. Cells were washed twice with ice-cold FACS buffer and analyzed using a FACSCalibur and CELLQuest software (BD Biosciences).

RESULTS

Generation of μ2 mutant mice. To generate μ2 mutant mice by targeted gene disruption, the genomic fragment containing exons 3 to 11 of the μ2 gene was replaced with a *neo* gene by homologous recombination in ES cells. Southern blot analysis using the 5′ and 3′ probes indicated in Fig. 1 of G418- and ganciclovir-resistant ES clones confirmed the homologous recombination event. The resulting $\mu2^{+/-}$ cells were injected into C57BL/6 blastocysts to establish chimeric mice. Further crossing of chimeric mice with C57BL/6 mice was carried out to obtain heterozygous ($\mu2^{+/-}$) mutant mice. $\mu2^{+/-}$ mice had an apparently normal phenotype; they were healthy and fertile and had a normal life span.

Early embryonic lethality in $\mu2^{-/-}$ mice. $\mu2^{+/-}$ mice were intercrossed to obtain homozygous mutant mice for the μ2 gene disruption ($\mu2^{-/-}$). The genotype of the offspring was determined at 4 weeks after birth by Southern blot analysis (Fig. 1). It revealed that all the progeny were either $\mu2^{+/+}$ (i.e., wild type) or $\mu2^{+/-}$, without any $\mu2^{-/-}$ mutants (Table 1). The ratio of $\mu2^{+/+}$ to $\mu2^{+/-}$ mice followed Mendelian expectations.

We next determined at what stage of development the $\mu2^{-/-}$ embryos died. We examined their genotypes at E10.5 and E3.5 of gestation. As shown in Table 1, we found only wild-type and $\mu2^{+/-}$ blastocysts, but no $\mu2^{-/-}$ blastocysts, suggesting that $\mu2^{-/-}$ mice die early in gestation.

We also analyzed ES cells cultured from blastocysts obtained after intercrossing of the $\mu2^{+/-}$ mice. All of the ES cells developed from the blastocysts were either $\mu2^{+/+}$ or $\mu2^{+/-}$ (data not shown). These results support the conclusion that there were no $\mu2^{-/-}$ embryos at the E3.5 stage. In addition, repeated attempts to establish $\mu2^{-/-}$ ES cells in culture were unsuccessful (data not shown). Therefore, we conclude that μ2 is essential for early embryonic development and, most likely, for the survival of ES cells.

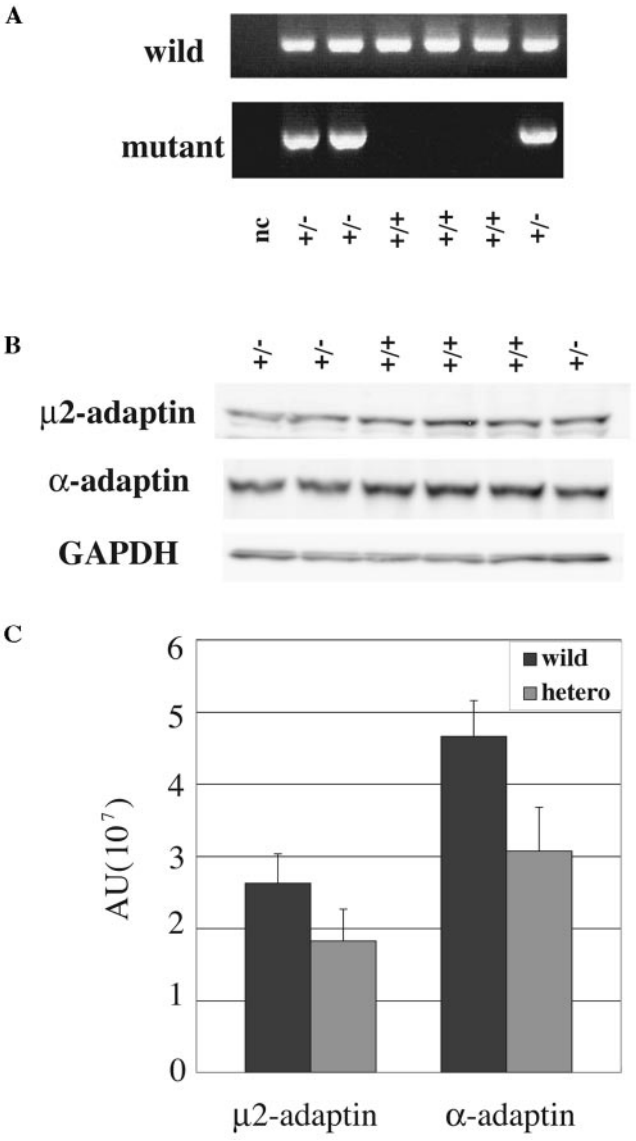

FIG. 2. Expression of μ2 and α proteins in $\mu2^{+/-}$ and $\mu2^{+/-}$ MEFs. (A) Genotyping of MEFs by PCR. These cell lines were established from embryos obtained by crossing wild-type females with heterozygous males. The resulting genotypes are indicated below each lane. Distilled water was used as a template for the negative control (nc; lane 1). (B) Western blot analysis of μ2 and α proteins in $\mu2^{+/-}$ and $\mu2^{+/-}$ MEFs. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control for the amount of protein in each lane. (C) The results shown in panel B were quantified. The amount of protein in each lane was corrected by the amount of GAPDH. Results are expressed as mean ± standard deviation from three experiments.

Expression of AP-2 subunits in $\mu2^{+/-}$ MEFs. To investigate the effect of the heterozygous ablation of the μ2 gene on the protein expression level of AP-2 and on its cellular function,

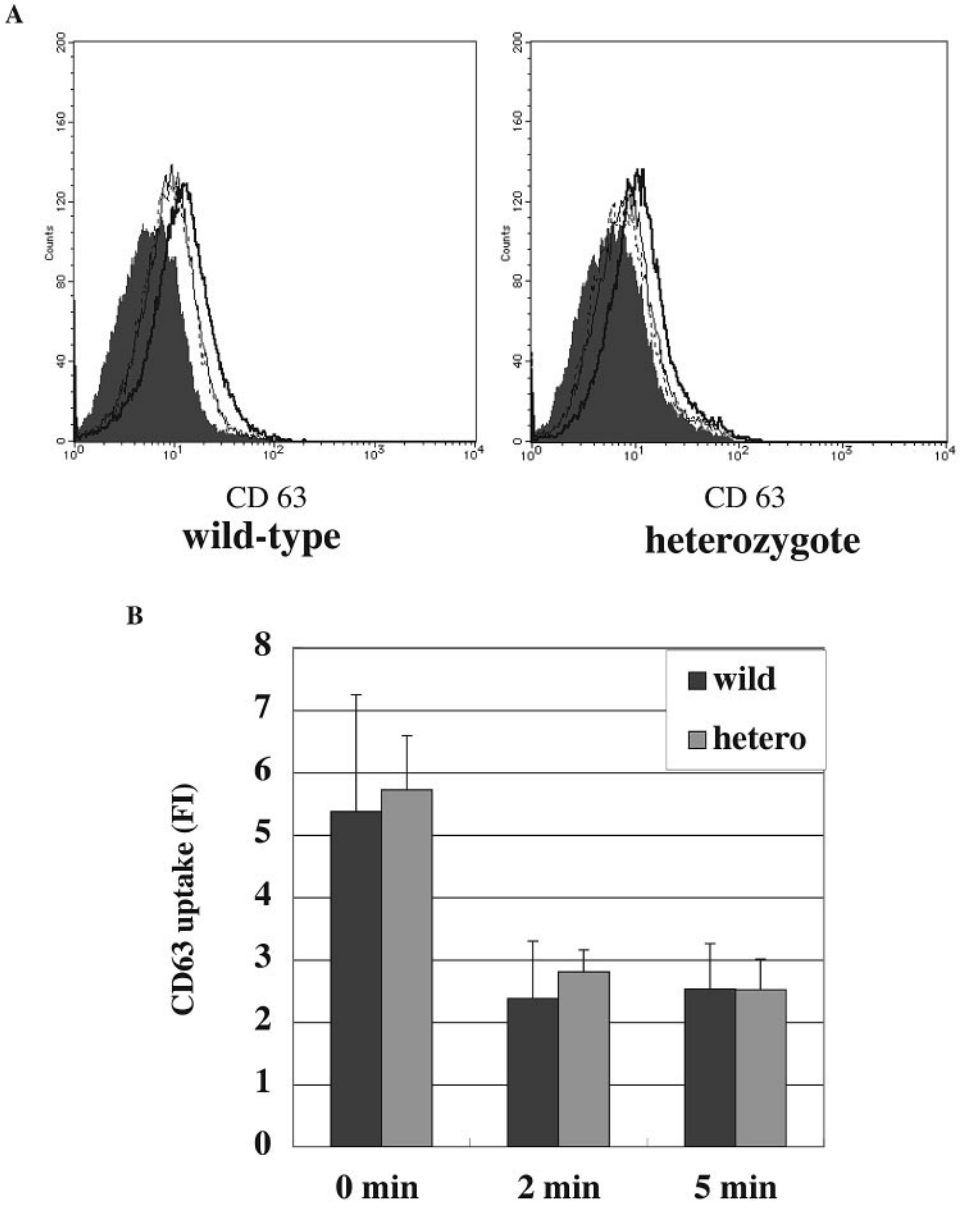

FIG. 3. Kinetics of the internalization of CD63 in MEFs. MEFs were allowed to internalize an antibody to mouse CD63 for the indicated times (0, 2, or 5 min) at 37°C. This was followed by staining with secondary antibody to detect the anti-CD63 antibody remaining on the cell surface by FACS analysis. (A) Histograms showing CD63 expression on wild-type (left bar) and heterozygote (right bar) MEFs. The thin solid line, thick solid line, and dotted line represent surface expression at 0, 2, and 5 min of incubation, respectively. The gray curve corresponds to a negative staining control. (B) Kinetics of CD63 internalization. Data represent the amount of anti-CD63 antibody remaining on the surface after the indicated incubation times. FI, fluorescent intensity. Results are expressed as mean ± standard deviation from three experiments.

we first established $\mu2^{+/+}$ and $\mu2^{+/-}$ MEFs from littermates. There were no obvious differences between $\mu2^{+/+}$ and $\mu2^{+/-}$ cells in terms of appearance and growth rate (data not shown). The levels of AP-2 $\mu2$ and α subunits in MEFs were determined by Western blot analysis. As shown in Fig. 2, the amount of $\mu2$ in $\mu2^{+/-}$ MEFs was lower than that in $\mu2^{+/+}$ MEFs ($P = 0.08$, Student's t test), albeit the decrease was less than half, as could have been expected for a heterozygous mutant. A similar result was obtained for the α subunit ($P = 0.02$, Student's t test). This reduction in α levels is consistent with previous studies indicating that AP complexes are unstable when one of the subunits is missing (7, 19, 30).

Endocytosis in $\mu2^{+/-}$ MEFs. Next, we examined the endocytosis of the tetraspanin, CD63, a lysosomal membrane protein. CD63 is transported to late endosomes or lysosomes by a pathway that is partly dependent on AP-3 (7, 36). A small amount of CD63 is also detected on the plasma membrane, and these molecules are internalized via AP-2-mediated endocytosis (18). This is in line with the presence of a YXXΦ-type motif in the C-terminal cytosolic tail of CD63, which interacts

with the μ2 and μ3 subunits of AP-2 and AP-3, respectively (7, 17, 41).

To test whether heterozygous ablation of the μ2 gene affected the internalization rate of CD63, MEFs were incubated with an antibody to mouse CD63 at 4°C. After washing, the antibodies were allowed to internalize at 37°C for different periods, and the amount remaining at the cell surface was determined by FACS analysis (Fig. 3). We observed that the amount of CD63 on the surface of $\mu2^{+/-}$ MEFs was comparable to that on the surface of $\mu2^{+/+}$ MEFs at the start of internalization (i.e., 0 min), suggesting that the steady-state level of CD63 on the plasma membrane was not affected by ablation of one copy of the μ2 gene. After 2 min of incubation, approximately half of the antibodies were internalized in both $\mu2^{+/-}$ and $\mu2^{+/+}$ MEFs. These data indicated that the heterozygous mutation of μ2 did not alter the internalization rate of CD63.

DISCUSSION

It is well established that AP-2 is crucial for receptor-mediated endocytosis in cultured cells (45). However, the physiological requirement for AP-2 in mammalian organisms remained to be assessed. To this end, we inactivated the μ2 gene in mice. The resulting $\mu2^{+/-}$ mice were viable and normal by many criteria. However, no $\mu2^{-/-}$ mice were born from heterozygous intercrossing, suggesting that $\mu2^{-/-}$ mice were embryonic inviable. Further analysis revealed that $\mu2^{-/-}$ homozygous mice died before the E3.5 blastocyst stage. The most likely explanation for the early death of $\mu2^{-/-}$ embryos is that maternally inherited μ2 mRNA becomes too dilute at the stage of cleavage division around E3.5 to support further development. Mice deficient for several other genes have also been reported to be lethal around E3.5 (32, 46). These observations, in addition to the failure to obtain $\mu2^{-/-}$ ES cells in culture, suggest that the AP-2 complex is indispensable for cell viability.

We have previously shown that μ subunits directly recognize YXXΦ-type sorting signals within the cytoplasmic tails of transmembrane proteins (33). Thus, μ subunits of the AP complexes play a pivotal role in AP complex-driven protein sorting (3). Mice lacking genes encoding μ subunits of other AP complexes have been established and analyzed. μ1A-deficient embryos die around E13.5 due to hemorrhage, indicating that μ1A is dispensable for cell viability but required for development of the embryo (26). In contrast, disruption of the gene encoding the other subunit of AP-1, γ1, in mice results in early embryonic lethality (48). The phenotypic difference between these two mutant mice may be explained by the compensation for the μ1A deficiency by the closely related isoform μ1B, which is specifically expressed in epithelial cells (9, 34, 39). Indeed, exogenously expressed μ1B partially rescued the defective sorting of mannose 6-phosphate receptors in MEFs prepared from μ1A-deficient mice (8, 25). Given that μ1B is detected by reverse transcription-PCR in ES cells (F.N., N.S., and H.O., unpublished data) and that the majority of cells belong to epithelial lineages early in embryogenesis, it is likely that μ1B substitutes for μ1A until E13.5, when organogenesis proceeds to develop organs lacking μ1B expression, such as the brain and spinal cord. In contrast to μ1A, no other subunit seems capable of substituting for γ1.

We have recently developed mice deficient in the μ3B subunit of the neuron-specific AP-3B complex and demonstrated that they survive but suffer from a neuronal disorder (30).

Regarding μ2, we and others have reported the use of small interfering RNAs (siRNAs) to reduce expression of this protein (16, 18, 24, 28). The μ2 RNAi-treated cells remained viable, although endocytosis of TfR (16, 18, 24, 28) and lysosome-associated membrane proteins was severely impaired (18). Likewise, overexpression of the adaptor-associated kinase AAK1 and a dominant-negative mutant of μ2 unable to bind YXXΦ-type signals decreased internalization of the TfR (5, 31). Nonetheless, the cells overexpressing AAK1 or mutant μ2 also appeared viable. It is thus likely that the small amount of μ2 remaining in the RNAi-treated cells or incomplete inhibition by the dominant-negative constructs still allows for the maintenance of cell viability. A similar observation has been made for clathrin. RNAi knockdown of clathrin heavy chain drastically inhibits TfR endocytosis but apparently does not lead to cell death (13, 16, 18, 24, 27, 28). In contrast, ablation of the clathrin heavy chain gene in the chicken DT40 B-cell line leads to apoptosis (47).

As mentioned above, the amount of AP-2 is only moderately decreased in $\mu2^{+/-}$ MEFs compared to that in $\mu2^{+/+}$ MEFs. Consistent with this observation, no apparent difference was observed in the kinetics of internalization for CD63, a lysosomal membrane protein that is internalized in a YXXΦ signal-dependent (17, 23, 41) and AP-2-dependent (18) fashion. These results suggest that heterozygous mutation of μ2 did not affect its cellular function in vivo as well as in vitro.

In conclusion, our study provides the first piece of evidence that AP-2 is indispensable for early embryonic development in mammals, most likely due to its requirement for cell viability.

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